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(54) Title: GENE MUTATED IN WOLFRAM SYNDROME
(54) Titre: MUTATION DE GENE ASSOCIEE AU SYNDROME DE WOLFRAM

(57) Abstract

This invention provides a novel gene, WFS1, isolated from human chromosome 4p. Mutation of the WFS1 gene is associated with the development of Wolfram Syndrome. The WFS1 gene, along with cDNAs, encoded protein and antibodies immunologically specific for the protein, provide a biological marker for early diagnosis of the syndrome, and for predicting predisposition of an individual for the syndrome. The gene also will be useful in gene replacement therapy, or for development of new methods and agents for treating Wolfram Syndrome.

(57) Abrégé

L'invention se rapporte à un nouveau gène, WFS1, que l'on a isolé sur le chromosome humain 4p. La mutation de ce gène WFS1 est associée au développement du syndrome de Wolfram. La protéine codée par ce gène WFS1, associé à des séquences d'ADNc, ainsi que des anticorps spécifiques de ladite protéine d'un point de vue immunologique fournissent un marqueur biologique permettant un diagnostic précoce du syndrome ainsi qu'un diagnostic de la prédisposition d'un sujet à développer ce syndrome. Ce gène peut également être utilisé en thérapie génique de remplacement ou pour le développement de nouvelles méthodes et de nouveaux agents permettant de traiter le syndrome de Wolfram.

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(57) Abstract

This invention provides a novel gene, *WFS1*, isolated from human chromosome 4p. Mutation of the *WFS1* gene is associated with the development of Wolfram Syndrome. The *WFS1* gene, along with cDNAs, encoded protein and antibodies immunologically specific for the protein, provide a biological marker for early diagnosis of the syndrome, and for predicting predisposition of an individual for the syndrome. The gene also will be useful in gene replacement therapy, or for development of new methods and agents for treating Wolfram Syndrome.

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Description

GENE MUTATED IN WOLFRAM SYNDROME

FIELD OF THE INVENTION

This invention relates to the field of diagnosis and treatment of certain forms of diabetes. In particular, this invention provides a novel gene, the disruption of which is associated with Wolfram Syndrome, and methods of using the gene and specific mutants thereof as diagnostic tool for prediction and early detection of the syndrome.

BACKGROUND OF THE INVENTION

Various scientific and scholarly articles are referred to in brackets throughout the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains.

Wolfram syndrome (WFS)(OMIM 222300) was first described in 1938 as a combination of familial juvenile-onset diabetes mellitus and optic atrophy. Other clinical features subsequently emerged and accordingly, WFS is also referred to as the DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness) syndrome. Most patients with this

progressive disorder eventually develop all four cardinal manifestations, and die prematurely with widespread atrophic changes throughout the brain. Insulin-requiring diabetes mellitus occurs with mean age of onset at 6-8 years. When examined, pancreatic islets display atrophic and insulin-producing β-cells selectively absent. The disease is believed to account for 1/150 patients with young-onset insulin-requiring diabetes mellitus.

The pathogenesis of Wolfram syndrome is unknown. Diagnosis is usually made in offspring of unaffected often-related parents, suggesting autosomal recessive inheritance. Linkage of the gene to markers on chromosome 4p (Polymeropoulos et al., Nature Genetics 8: 95-97, 1994) was reported, and later confirmed (Collier et al., Am L Hum Genet 59: 855-863, 1996). Recombinants placed the gene in an interval between markers 5.5 cM apart.

Isolation and characterization of the gene or genes associated with Wolfram Syndrome is vital for the prediction, diagnosis and, ultimately, treatment of the disease. Currently there is no way of knowing for sure if an individual is predisposed to Wolfram Syndrome, particularly children too young to have developed the characteristic symptoms. Early diagnosis may lead to effective methods of treatment. Identification and isolation of the gene or genes associated with Wolfram Syndrome would further enable the development of screening procedures to assist in genetic counseling, as well as enabling detailed study of its function and subsequent development of therapeutic methods or agents.

SUMMARY OF THE INVENTION

In accordance with the present invention, a gene is provided, whose mutation is highly correlated with Wolfram Syndrome in humans. The symptoms of Wolfram

Syndrome consist of diabetes insipidus, diabetes mellitus, optic atrophy and deafness. This gene is found in human genome between markers *D4S500* and *D4S431* on chromosome 4p, and is referred to herein as *WFS1*. One variant of *WFS1* is found in SEQ ID NO:1.

Another aspect of the invention comprises several isolated nucleic acids from the human and mouse *WFS1* genes. In a preferred embodiment, these nucleic acids are the genomic sequence of the *WFS1* gene from human (SEQ ID NO:1), a cDNA sequence from the human *WFS1* gene (SEQ ID NO:2), and a cDNA from the mouse *WFS1* gene (SEQ ID NO:4). In another preferred embodiment, the isolated nucleic acids are substantially the same or 60% homologous to SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4. In yet another preferred embodiment, the nucleic acids encode SEQ ID NO:3 and SEQ ID NO:5, substantially the same variants of SEQ ID NO:3 and SEQ ID NO:5, variants with at least 60% homology to SEQ ID NO:3 and SEQ ID NO:5, and variants of SEQ ID NO:3 with the mutations and polymorphisms listed in Table 1. In a final preferred embodiment, the isolated nucleic acids are oligonucleotides (SEQ ID Nos:6-41) that have been designed based on SEQ ID NO:1.

In accordance with another aspect of the invention, a selection of isolated polypeptides is provided, which result from the expression of part or all of the human and mouse *WFS1* genes. In one preferred embodiment, the polypeptides are encoded by SEQ ID NO:3 and SEQ ID NO:5. In another preferred embodiment, the polypeptides are substantially the same as SEQ ID NO:3 and SEQ ID NO:5, or at least 60% homologous to SEQ ID NO:3 and SEQ ID NO:5.

In accordance with another aspect of the invention, a selection of antibodies that are immunologically specific to the aforementioned

polypeptides is provided.

In accordance with another aspect of the invention, a series of methods is provided, which use the *WFS1* gene to genetically characterize mammalian subjects. In a preferred embodiment, the *WFS1* gene is used to make labeled probes which are used to detect *WFS1* nucleic acids. In another preferred embodiment, variant forms of *WFS1* are sequenced, compared to the sequence of *WFS1*, and mutations and polymorphisms determined. In a particularly preferred embodiment, human genomic DNA is sequenced and compared to SEQ ID NO:1. In a very particularly preferred embodiment, the primers in Table 2 are used to sequence the variant human gene. In yet another preferred embodiment, restriction enzymes are selected that differentially digest the wild type and variant gene, *WFS1* nucleic acids are digested and separated by size, and the *WFS1* nucleic acids are detected. In a particularly preferred embodiment, the human DNA is digested, and the primers in Table 2 are used to amplify the DNA before digestion.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed descriptions and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Pedigrees of Wolfram syndrome families, with individuals designated by disease status (bold-lined symbols affected, thin-lined symbols unaffected), and with derived haplotypes of chromosome 4p markers. Bold, underlined numbers represent disease-associated chromosomes. Italicized, underlined numbers refer to disease-associated chromosomes with historical recombinants. Consanguineous Japanese families: Fig. 1a, WS-1; Fig. 1b, WS-2; Fig. 1c,

WS-3 (an affected daughter, not shown, was deceased before initiation of the study). Caucasian families: Fig. 1D, WS-4; and Fig. 1E, WS-5. Markers were ordered from telomeric to centromeric as described in Example 1.

Figure 2. Physical map of the Wolfram syndrome critical region. Fig. 2A: The horizontal line at the top of the figure represents a portion of chromosome 4p, with the centromere to the right and pter to the left. The dashed line represents the interval of *D4S500-D4S431*, the critical region of the Wolfram syndrome gene. P1 and BAC clones are represented as lines. Their length reflects the number of STSs and not the actual size. The name of each clone is given to the right of the line. Marker names are noted above the line and correspond to the symbols on the line. Fig. 2B: An expanded schematic of the genomic structure of the Wolfram gene, *WFS1*, with exons indicated by boxes. The entire gene is encompassed within a 33.4 kb region.

Figure 3. Expression of *WFS1* mRNA in adult tissues. Fig. 3A: Northern analysis with human adult polyA+ RNA (5 μg) derived from various tissues was hybridized with a ^{32}P-labeled 854-bp genomic fragment of the *WFS1* gene. Fig. 3B: Re-hybridization of the blot with ^{32}P-labeled β-actin cDNA as a loading control. Fig. 3C: Northern analysis with human adult total RNA (20 μg) hybridized with ^{32}P-labeled *WFS1* cDNA. Fig. 3D: Re-hybridization of the blot in c. with ^{32}P-labeled ribosomal cDNA as a loading control.

Figure 4. Hydrophobicity analysis was conducted by the method of Kyte and Doolittle (J Molec Biol 157: 105-132, 1982) using a window size of 9 amino acid residues. Average hydrophobicity values are plotted as a function of position along the polypeptide chain.

Figure 5. Comparison of human and mouse *WFS1*

protein sequences. Plain text indicate identical residues. Amino acid gaps between human and mouse proteins are shown by dashes. The locations of the mutations found in Wolfram syndrome patients, 3 missense, the premature stop codon (X), 2 deletions, and the 7 bp repeat insertion, are indicated below the sequences. A predicted prenyltransferase α-subunit repeat structure (A493 to L502) is underlined.

Figure 6. Co-segregation of *WFS1* mutations with the disease phenotype in Wolfram syndrome families. Gig. 6a: The 1685del(N) in family WS-2, showing that each affected child (III-1, -2, -3, and -4) is homozygous for the mutation. Fig. 6b: Sequence chromatograms of the region of exon 8 showing the 15 bp deletion in a patient homozygous for the mutation, along with a normal control. Fig. 6c: Segregation of the 1681C to T and the microscopic deletion in family WS-5. The 1681C to T mutation destroys a BsmF1 site in a 766 bp PCR (polymerase chain reaction) fragment. The hemizygous T(-) affected children (II-1, -2, and -4) have only a 766 bp uncut fragment, while the hemizygous C(-) mother (I-4) and unaffected daughter (II-3) have 686 bp and 80 bp bands, and the heterozygous CT father (I-3) has 766 bp, 686 bp and 80 bp bands. The symbols "-" and "+" refer to the absence or presence of enzyme.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

With reference to nucleic acids molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that

is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to proteins or peptides, the term "isolated protein (or peptide)" or "isolated and purified protein (or peptide)" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the

compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. In the comparisons made in the present invention, the BLAST programs (NCBI) and parameters used therein were employed, and the DNAstar system (Madison, WI) was used to align sequence fragments of genomic DNA sequences. However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in

determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides or other single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally

used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the

transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA

that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, a heterologous region is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein. The term "DNA construct", as defined above, is also used to refer to a heterologous region, particularly one constructed for use in transformation of a cell.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Description

In accordance with the present invention, a novel human gene, *WFS1*, has been isolated. The inheritance of a mutated *WFS1* gene is highly correlated

with the development of Wolfram Syndrome in the human population. Normal function of *WFS1* appears to be essential for survival of pancreatic islet β-cells and neurons. Included in the invention is the method for using the gene sequence to genetically screen for presence of the potentially mutated forms of the gene for diagnosis and prognosis of the disease in patients.

The *WFS1* gene in humans spans 33.4 kb on chromosome 4p and is composed of 8 exons (Fig. 2B; SEQ ID NO:1). The ~5kb of sequence upstream of the start of *WFS1* open reading frame is expected to contain one or more transcriptional or translational regulatory elements. The *WFS1* human cDNA is 3.688 kb long (SEQ ID NO:2) and encodes a predicted protein 890 residues long with a predicted molecular mass of 100.29 kDa (SEQ ID NO:3). Comparison of the *WFS1* cDNA sequence with those in public databases found no related genes. A mouse cDNA of *WFS1* has also been isolated. The mouse cDNA (SEQ ID NO:4) is 3511 nucleotides long and has 83.9% nucleotide identity to the human gene. The predicted protein sequence encoded by the mouse cDNA (SEQ ID NO:5) has a 86.1% amino acid similarity to the predicted human *WFS1* protein.

The inventors have isolated the *WFS1* gene represented by SEQ ID NO:1 from the human genome by positional cloning. Previous work had isolated the Wolfram gene between markers *D4S432* and *D4S431* on chromosome 4p, 5.5 cM (~5500 kb) apart. In the development of this invention, five families with individuals having typical Wolfram syndrome phenotypes were genotyped with genetic markers shown to locate between *D4S432* and *D4S431* by physical and/or radiation hybrid mapping (see Example 1). The region containing the Wolfram gene was thus narrowed further to a region

between *D4S500* and *D4S431*. The critical region between *D4S500* and *D4S431* was estimated to be <250 kb as determined by contig mapping of BAC and P1 genomic clones. Three clones were sequenced and the sequence of much of the contig region was determined.

Exon trapping of two BAC clones was employed to generate expressed sequence tags (ESTs) of the region and then to determine areas with open reading frames that would be likely to contain a gene. Among a number of ESTs isolated was one predicted to be the 3' end 1.8 kb exon of a gene. A genomic fragment of this region hybridized to a 3.7 kb RNA on a Northern blot. The gene was determined to be expressed in all tissues, but surprisingly was most abundant in pancreatic islets compared to that in the exocrine pancreas. The abundance of *WFS1* RNA in pancreatic islets was particularly revealing because one of the outcomes Wolfram Syndrome is atrophy of pancreatic islets.

A full length cDNA clone of *WFS1* was obtained by screening a human infant brain cDNA library (SEQ ID NO:2). This clone was 3,688 nucleotides long and contained an appropriate start methionine, open reading frame, and polyadenylation signal. Comparison of the cDNA sequence of *WFS1* with those in public databases revealed no related genes. Translation of the cDNA sequence predicts a polypeptide of 890 amino acid residues with a molecular mass of 100.29 kDa. The protein is distinguished grossly by the presence of 3 structural domains, a hydrophilic N-terminal region of ~300 residues, a hydrophilic C-terminal region of ~240 residues, and a central hydrophobic core of ~350 residues. Inspection of the hydrophobicity curve suggests the presence of ~10 transmembrane segments.

When patients with Wolfram Syndrome were checked for mutagenesis in the *WFS1* gene, seven mutations

were found in the full-length clones derived from the original EST. PCR (polymerase chain reaction) was used to amplify exons of *WFS1* from subjects with Wolfram Syndrome, and the products were sequenced. Comparison of these sequences with the wild type gene revealed probable loss of function mutations in all cases, as described in greater detail below.

The following description set forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (herein "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) (herein "Ausubel et al.") are used. Unless otherwise specified, general genome analysis procedures were used, such as set forth in Genome Analysis: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1997).

I. Preparation of *WFS1* nucleic acid molecules, encoded proteins and immunologically specific antibodies

A. Nucleic Acid Molecules

Nucleic acid molecules comprising part or all of the *WFS1* gene of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information, such as Sequence I.D. Nos. 1, 2 and 4, enables preparation of an isolated nucleic acid molecule

of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified by high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a double-stranded DNA molecule several kilobases in length may be synthesized as multiple smaller segments of appropriate complementarily. Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

WFS1 nucleic acid sequences may be isolated from appropriate biological sources using methods known in the art. In a preferred embodiment, a human genomic clone is isolated from a human genomic P1 library. In another preferred embodiment, a cDNA clone is isolated from the Marathon-Ready human fetal brain λ gt10 cDNA library (Clontech). In yet another preferred embodiment, a mouse cDNA is isolated from a mouse pancreatic β-cell line (MIN6) cDNA library. The isolation of human and mouse clones is not limited to the aforementioned libraries, and other commercially available human and mouse libraries may be used. Alternatively, cDNA or genomic clones from other species may be obtained.

In accordance with the present invention, nucleic acids having the appropriate sequence homology

with part or all of Sequence I.D. Nos. 1, 2 or 4 may be identified by using hybridization and washing condition of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5 x SSC, 5 x Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hour. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2 x SSC and 1% SDS; (2) 15 minutes at room temperature in 2 x SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1 x SSC and 1% SDS; (4) 2 hours at 42-65°in 1 X SSC and 1% SDS, changing the solution every 30 minutes. In a preferred embodiment, hybridizations are performed in hybridization solution comprising 0.5 M $NaPO_4$, 2 mM EDTA, 7% SDS and 0.1% sodium pyrophosphate (pH 7.1) at about 65°C for 20 hours. For high-stringency conditions, membranes are subsequently washed sequentially for 1 hour each in: (1) 2X SSC, 0.5X SET, 0.1% sodium pyrophosphate; and (2) 0.1X SSC, 0.5X SET, 0.1% sodium pyrophosphate. For low-stringency conditions, membranes are washed at 50°C for 30 minutes in 2X SSC, 0.5X SET, 0.1% sodium pyrophosphate.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6\text{Log}[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1%

decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C, and wash in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C, and wash in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C, and wash in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, genomic clones are maintained in a COS-7 cells in the vector pSPL3 (Life Technologies, Inc.). In another preferred embodiment, PCR products are subcloned into pAMP10 using the UDG cloning kit (GIBCO

BRL), and propagated in a suitable *E. coli* host cell. In another preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pGEMT (PROMEGA), and propagated in *E. coli*.

WFS1 nucleic acid molecules of the invention (including those containing known polymorphisms and mutations) include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of Sequence I.D. Nos. 1, 2 and 4. Such oligonucleotides are useful as probes for detecting *WFS1* genes (and specific mutations) in test samples, e.g. by PCR amplification, or as potential regulators of gene expression.

B. Proteins and Antibodies

A full-length *WFS1*-encoded protein of the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by immunoaffinity purification. However, due to the limited amount of such a protein that may be present in a sample at any given time, particularly in tumors or tumor cell lines, conventional purification techniques are not preferred in the present invention.

The availability of the isolated *WFS1* coding sequence enables production of protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro*

transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, the recombinant protein may be produced by expression in a suitable procaryotic or eukaryotic system. For example, part or all of a DNA molecule, such as the cDNA having SEQ ID NO:2 or No. 4, may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the bacterial host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The protein produced by *WFS1* gene expression in a recombinant procaryotic or eukaryotic system may be purified according to methods known in the art. A commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

Proteins prepared by the aforementioned methods may be analyzed according to standard procedures. For

example, such proteins may be subjected to amino acid sequence analysis, according to known methods.

Included in the present invention are antibodies capable of immunospecifically binding to proteins of the invention. Polyclonal antibodies directed toward *WFS1*-encoded proteins may be prepared according to standard methods. Monoclonal antibodies may be prepared, which react immunospecifically with various epitopes of the proteins. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. Polyclonal or monoclonal antibodies that immunospecifically interact with *WFS1*-encoded proteins can be utilized for identifying and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules. Other uses of antibodies are described below.

II. Uses of *WFS1* Nucleic Acids, Encoded Proteins and Immunologically Specific Antibodies

A. *WFS1* Nucleic Acids

Nucleic acids comprising part or all of the *WFS1* gene may be used for a variety of purposes in accordance with the present invention. As illustrated in Example 1, selected *WFS1* sequences (DNA, RNA or fragments thereof) may be used as probes to identify mutations or rearrangements in a patient's DNA, and/or monitor the level of *WFS1* transcripts in tissues. As discussed earlier, *WFS1* mutations are associated with the occurrence of Wolfram Syndrome, a disease of autosomal recessive inheritance. Early identification of patients

destined to develop Wolfram Syndrome may lead to preventive therapies. Identification of heterozygous individuals that are at risk of having a child with Wolfram Syndrome will be very useful in genetic counseling.

WFS1 sequences may be utilized as probes in a variety of assays known in the art, including but not limited to: (1) *in situ* hybridization; (2) Southern hybridization; (3) northern hybridization; and (4) assorted amplification reactions, such as polymerase chain reaction (PCR). In a preferred embodiment, large deletion and premature termination mutations are detected by separation on acrylamide or agarose gel electrophoresis and Southern blotting with probes made from *WFS1* gene sequences. Knowledge of the wildtype sequence allows the identification of point mutations in non-functional *WFS1* genes. In another preferred embodiment, mutated genes are differentiated from wildtype genes by using restriction enzyme sites that appear or disappear as the result of the mutation. *WFS1* nucleic acids are digested and the fragments are then separated and probed as described above.

Both of the above-mentioned preferred embodiments are illustrated in Example 1. The human genomic *WFS1* sequence was used to design screening procedures to quickly screen all the individuals in a Wolfram Syndrome patient's extended family. In the case of large deletion mutations, the screen entailed amplifying the region of the gene encompassing the deletion, then separating the products by agarose gel electrophoresis and performing Southern blot hybridization using a labelled wild type PCR fragment. In the case of point mutations, the PCR primers were designed as above, the mutant and wildtype products were digested with a restriction enzyme that was specific to

either the sequence at the point mutation or the corresponding wildtype sequence. The products of the digestion, along with undigested control nucleic acids, were separated and detected as above. In all of the six extended families studied, inheritance of a homozygous complement of a mutated *WFS1* gene was consistent with the development of the disease and the pedigree of the family. Of particular interest are the three mis-sense mutations in the human *WFS1* predicted polypeptide, whose wild type sequence is found to be conserved in the predicted mouse polypeptide. This sequence conservation, together with the mutational effect of non-conservative amino acid substitutions at these sites, suggests that these amino acids are critical for gene function. A primary screen of these sequences is a useful way to expedite the identification of mutations. Other critical mis-sense mutations may also be useful in connection with this invention. These other mutations can be found using procedures detailed in Example 1 and others well known in the art.

The *WFS1* nucleic acids of the invention may also be utilized as probes to identify related genes either from humans or from other species. In a preferred embodiment, a cDNA has been isolated from mouse insulinoma cDNA library by standard screening methods and RACE PCR. The mouse cDNA was 3,511 nucleotides long with 83.9% nucleotide identity to the coding sequence of the human gene and 86.1% predicted amino acid similarity. While the cloning of the mouse cDNA is illustrated in Example 1, those skilled in the art will appreciate that the isolation of *WFS1* from other species is by no means limited to mouse. Other mammalian species of interest include, but are not limited to, cow, cat, dog, horse, pig and rat. As is well known in the art, hybridization stringency may be adjusted so as to allow hybridization

of nucleic acid probes with complementary sequencing of varying degrees of homology.

The cDNA from the mouse *wfs1* gene is very useful because it allows further study of the gene and Wolfram Syndrome in system more conducive to research than human. The mouse clone may be used to create a targeting construct, which can be used for the targeted mutagenesis of the endogenous mouse gene. By creating both null and site-directed mutants, a mouse model system for Wolfram Syndrome can be generated. This mouse system can subsequently be used to elucidate the cellular function of the Wolfram gene product, as well as assist in developing therapies for the syndrome. Systems other than mouse can also be used to advantage. These systems include, but are not limited to animal models developed in mouse, various cultured human and mammalian cell systems (e.g., mouse and rat insulinoma cells) and frog oocyte expression systems.

As described above, the coding region of *WFS1* may also used to advantage to produce substantially pure *WFS1* encoded proteins or selected portions thereof. As described below, these proteins may also be used in diagnosis and therapy of Wolfram Syndrome.

B. Proteins and Antibodies

The *WFS1*-encoded protein, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies, which also may serve as sensitive detection reagents for the presence and accumulation of the *WFS1*-encoded polypeptide in cultured cells or tissues from living patients (the term "patient" refers to both humans and animals). Because the *WFS1*-encoded protein has not yet been isolated from natural sources, such antibodies will greatly accelerate the identification, isolation and characterization of this protein in mammalian cells and

tissues. Recombinant techniques enable expression of fusion proteins containing part or all of the *WFS1*-encoded protein. The full-length protein or fragments of the protein may be used to advantage to generate an array of monoclonal antibodies specific for various epitopes of the protein, thereby potentially providing even greater sensitivity for detection of the protein in cells or tissues. Monoclonal antibodies specific to variant portions of the *WFS1* polypeptide may also be used to advantage in diagnosing presence of a variant form of the gene.

Polyclonal or monoclonal antibodies immunologically specific for the *WFS1*-encoded protein may be used in a variety of assays designed to localized and/or quantitate the protein. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization of the protein in cultured cells or tissues; and (3) immunoblot analysis (e.g., dot blot, Western blot) of extracts from cells and tissues. Additionally, as described above, such antibodies can be used for the purification of *WFS1*-encoded proteins (e.g., affinity column purification, immunoprecipitation).

The following example is provided to describe the invention in greater detail. It is intended to illustrate, not to limit, the invention.

EXAMPLE 1
Isolation and Identification of Wolfram Gene and Mutants

METHODS

Patients and families. Three families originated from Japan: WS-1 (Nanko et al., Brit J Psychiatry 161: 282, 1992), WS-2 (Higashi, Am J Otology

12: 57-60, 1991, and WS-3 (Maruta et al., Clin Neurol 27: 725,732, 1987). Cell lines for the Caucasian family WS-4 were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. (family #1157). Family WS-5 is Caucasian Australian and WS-6 Saudi Arabian. Minimum criteria for diagnosis were young-onset insulin-dependent diabetes mellitus and progressive optic atrophy.

Microsatellite analysis. Initial genotyping was carried out as described previously (Nestorowicz et al., Hum Molec Genet 7: 1119-1128, 1998; Inoue et al., Diabetes 45: 789-794, 1996) with markers reported in the 1996 Genethon Microsatellite Map (*D4S127, Hox7, D4S412, D4S3023, D4S2925, D4S431, D4S2935, D4S3007, D4S394, D4S2983, D4S2923*). Second genotyping was performed with markers (*D4S2957, D4S2375, A348XA5, D4S827, D4S500, and D4S2366*), shown to locate in the interval of *D4S3023-D4S431* by physical and radiation hybrid mapping projects of chromosome 4. The STANFORD CHR 4 YAC MAP project and the CHROMOSOME 4 SUMMARY MAP were viewed at WEB sites (http://shgc.stanford.edu/, http://cedar.genetics.soton.ac.uk/). Primer sequences were obtained from the Genome Database (GDB - http://www.hgmp. mrc.ac.ok/gdb/gdbtop.html).

P1/BAC library screening. A human genomic P1 library (HD-K) was screened for clones containing *D4S500* and *D4S431* by PCR, using primers developed to specifically hybridize with those markers. Sequence-tagged sites (STSs) (SP6 and T7) from P1s 102C5, 89C1 and 77B6, as well as *D4S500* and *D4S431*, were used for BAC library screening by either PCR or by direct hybridization of library grid blots (Research Genetics, Inc., Huntsville, Alabama).

Exon-trapping, sample and shotgun megabase genomic sequencing. Restriction fragments from BAC 460K9 and 33H22 were cloned into the BamHI site of pSPL3,

transfected into COS-7 cells and spliced products obtained by RT-PCR were subcloned into pAMP10 using the UDG cloning kit (GIBCO BRL) and sequenced.

Sample and shotgun sequencing of BAC460K9 and 33H22 was accomplished as described (Wilson and Mardis, in *Genome Analysis: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, NY, 1997). Similarity searches with known genes and ESTs were performed using BLAST programs (NCBI). The sequencing project was compiled using LaserGene software (DNAStar, Madison, WI). Sequencing was performed on an ABI 373 with Prism dye terminator kits.

Northern blot analysis, cDNA screening, and 5' RACE analysis. A multiple adult human tissue polyA+ RNA Northern blot (Clontech- MTN human I) was probed with an 854-bp fragment (nt 2133-2986 *WFS1* cDNA) as described by the manufacturer. Total cellular RNA was isolated (Chomczynski and Sacchi, Anal Biochem 162: 156-159, 1987) and Northern analysis performed with 20 μg of RNA by hybridization with *WFS1* cDNA labeled with [α-^{32}P]dCTP as previously described (Ferrer et al., Diabetes 46: 386-392, 1997). RNA quality and loading was checked by staining the gel for ribosomal RNA and by hybridization with either β-actin or ribosomal RNA.

The same 854-bp fragment was used for screening an infant brain λ gt10 cDNA library. Six overlapping clones containing the *WFS1* gene were isolated. 5' RACE analysis was performed using Marathon-Ready human fetal brain cDNA (Clontech, Palo Alto, CA). The RACE products were subcloned into pGEMT vector (Promega Biotech, Madison WI) and sequenced. A mouse pancreatic β-cell line (MIN6) cDNA library was screened with human *WFS1* cDNA using standard reduced stringency conditions, and a 3.0 kb clone was isolated. A primer corresponding to the

predicted 5'-untranslated region, based on the mouse EST sequence (GenBank AA021827 and AA692227) that was homologous to the human cDNA, was synthesized and used for RT-PCR with MIN6 RNA (5'-CGGTTTCGGAGCAACTTCGC-3' , SEQ ID NO:42 and 5'- CACCTCAGCCTCGTTCTCAG -3', SEQ ID NO:43).

Mutation detection. M13 universal primer sequence was incorporated into the 5' terminus of primers for direct sequence analysis using an ABI automated DNA sequencer Model 373 (Chadwick et al., Biotechniques 20: 676-683, 1996). Primers used for exon amplification, genomic sequencing and mutation detection are as in Table 2.

The 2812del(TC) was detected with a labeled PCR fragment on a denaturing Long Ranger polyacrylamide gel (FMC Bioproducts). The 1685del(N_{15}) was detected by electrophoresis of PCR fragments on 4% agarose gels. The 2341C to T was detected by PCR, digestion with EcoNI (New England Biolabs (NEB)) and polyacrylamide-gel electrophoresis. The mutated allele was observed as 209 bp and 35 bp fragments.

The 2254G to T mutation was detected by PCR, digestion with AvaII (NEB) and agarose gel electrophoresis, with two bands (240 bp, 137 bp) in the wild allele, and three (240 bp, 120 bp, 17 bp) in the mutant. The 2114G to A mutation was detected by PCR, digestion with Tfi I (NEB) and agarose gel electrophoresis. The wild allele has one band of 528 bp, and the mutated allele has two bands (396 bp, 132 bp). The 1681C to T mutation destroys a BsmF1 restriction site, and primers are set 8a (Table 2), with resulting fragments described in Fig. 6c.

GenBank Accession Numbers. The human *WFS1* and

mouse *wfs1* cDNA sequences are deposited in GenBank: #AF084481 and #AF084482.

RESULTS

Linkage Analysis. Linkage studies were conducted on three Japanese families (WS-1, -2, and -3) and two Caucasian families (WS-4, -5) (Figs. 1A-1E), each with at least two individuals having typical Wolfram syndrome phenotypes. When genotyping with chromosome 4p markers used previously (Polymeropoulos et al., Nature Genetics 8: 95-97, 1994; Collier et al., Am J Hum Genet 59: 855-863, 1996), estimates for recombination fractions (θ) between WFS and the markers confirmed close linkage (lod =3.99 for *D4S431* at θ =0.05). Multipoint analysis with GENEHUNTER (version 1.1) (Kruglyak et al., Am J Hum Genet 59: 1347-1363, 1996) gave a lod >6.0 for the region encompassed by markers *D4S827 and D4S394*.

Haplotype Analysis and Mapping by Recombination. Haplotypes were constructed by inspection. The boundaries for the WFS gene had been defined by a telomeric recombinant at *D4S432* (Collier et al., 1996), and centromeric recombinants at *D4S431* (Polymeropoulos et al., 1994; Collier et al., 1996). Recombinants in families WS-1 - WS-6 were identified by genotyping with genetic markers shown to locate between *D4S432* and *D4S431* by physical and/or radiation hybrid mapping. In family WS-1, subject III-2, recombination of the telomeric region from *D4S827* was observed (Fig. 1A). In the Japanese family WS-2 (Fig. 1B), all affected subjects (III-1, -2, -3, and -4) were haploidentical centromeric to *D4S500*, suggesting the presence of a historical recombinant in the unrelated father (II-5). The centromeric boundary was confirmed as *D4S431* by a recombinant in WS-5, subject III-2 (Fig. 1E). We concluded that the WFS gene likely lies within the

interval *D4S500-D4S431*.

Construction of a genomic contig across the WFS critical region. In the STANFORD CHR 4 YAC MAP project, *D4S827* and *D4S431* are within two overlapping YACs encompassing the region (204H9 and 420E4). Further, *D4S500* and *D4S431* were located on the same YAC (420E4). A contig was constructed with genomic human P1/BAC clones, and a contiguous genomic map encompassing the WFS gene was obtained (Figure 2). STS content mapping confirmed overlapping clones. As the interval between *D4S500* and *D4S431* was covered by a P1 (102C5) and a BAC clone (33H22), the critical region was estimated to be <250 kb.

Identification of candidate genes within the *WFS* region, and cloning the *WFS1* gene. By exon trapping of BACs 33H22 and 460K9, an expressed sequence tag (EST) was found that resulted in the cloning of the γ-isoform of the B regulatory subunit of the human protein phosphatase 2A (PP2ABRγ). The genomic structure of PP2ABRγ was determined, and direct sequence analysis of probands excluded this gene. In parallel, we initiated large-scale genomic sequencing from BACs 460K9 and 33H22, and P1 102C5. A total of ~180 kb of sequence was analyzed. Among a number of EST matches, one was predicted to be the 3'-end exon containing a 1.8 kb open reading frame (exon 8, Fig. 2B). This EST was further evaluated as it was unambiguously within the critical region.

The size of the full-length mRNA and pattern of tissue expression was determined by PCR amplification of an 854-bp genomic fragment of the region that was hybridized to a multiple-tissue Northern blot with polyA+ RNA. A major transcript of ~3.7 kb was expressed in all tissues including pancreas (Fig. 3A). Northern analysis of total RNA (20 μg) revealed the gene most abundantly

expressed in pancreatic islets compared to that in exocrine pancreas (Fig. 3C).

A full-length clone was obtained by screening a human infant brain cDNA library. Six clones were isolated and subsequently 5' RACE analysis was performed. These analyses yielded a composite cDNA sequence of 3.688 kb. The longest open reading frame extended from nt 171 to 2843. The methionine at position 171 was chosen as the translation initiation codon primarily because it conforms to Kozak's rule (Kozak, Mamm Genome 7: 563-574, 1996). A consensus polyadenylation site (aataaa) was located at position 3615-20, 19 bases upstream from the polyA tail. The gene was named *WFS1*.

Predicted characteristics of the WFS1 protein, and cloning of the mouse cDNA. Comparison of the cDNA sequence of *WFS1* with those in public databases revealed no related genes. Translation of the cDNA sequence predicts a polypeptide of 890 amino acid residues with a molecular mass of 100.29 kDa. Hydrophobicity analysis of the deduced amino acid sequence is presented in Figure 4. The protein is distinguished grossly by the presence of 3 structural domains, a hydrophilic N-terminal region of ~300 residues, a hydrophilic C-terminal region of ~240 residues, and a central hydrophobic core of ~350 residues. Inspection of the hydrophobicity curve suggests the presence of ~10 transmembrane segments, if it is assumed that this region of the protein consists of α-helical segments. Comparison of the predicted amino acid sequence with entries in the Prosite database produced a single match to the prenyltransferase α-subunit repeat structure.

A mouse *wfs1* cDNA was isolated from a mouse insulinoma (MIN6) (Ishihara et al., Diabetologia 36: 1139-1145) cDNA library, and completed by RT-PCR. The mouse *wfs1* cDNA was 3511 nucleotides with 83.9%

nucleotide identity to the coding sequence of the human gene, and 86.1% amino acid similarity (Figure 5).

Genomic structure of the WFS1 gene and mutations in WFS patients. The genomic structure of *WFS1* was determined by comparison of cDNA and genomic sequences obtained by shotgun sequencing of BAC460K9 and 33H22 and sequences in the Stanford Human Genome Center database (http://www.shgc.stanford.edu). The gene was found to be composed of eight exons (Fig. 2B) in 33.4 kb of genomic DNA.

For mutation screening, exons were amplified and sequenced from patients' genomic DNA. A TC deletion at position 2812 for subject WS-1 III-2 predicted a frameshift at codon 882, designated del882fs/ter937 (Table 1), with absence of the normal stop codon at 891 and the introduction of a new downstream termination codon. The predicted *WFS1* protein contains 937 amino acids, 47 more than the normal protein. All 3 affected sibs (WS-1 III-1, -2, and -4) were homozygous for this mutation, while the unaffected sib and the parents were heterozygous, indicating a disease-specific mutation. The 2812delTC mutation was not found in 80 healthy control Japanese subjects (160 chromosomes, see Table 1).

In other WFS families, six additional mutations were found in exon 8 (Table 1). In family WS-2, affected offspring (III-1, -2, -3, and -4) inherited a 15 bp deletion resulting in del508YVYLL, homozygous by descent from related heterozygous parents. Co-segregation of this deletion with the WFS phenotype is shown in Fig 6A. A sequence chromatogram from an affected child homozygous for the 15 bp deletion is shown in Fig. 6B. In family WS-3, both affected offspring (II-1 and -2) were homozygous for a 2341 C to T transversion resulting in a P724L mutation. In the Caucasian family WS-4, all affected offspring (II-1, -3, and -4) were found to be compound heterozygotes for a 2254 G to T transition

resulting in a G695V (paternal) mutation, and a 2114 G to A transversion resulting in a W648X (maternal) mutation. The W648X mutation predicts a premature termination, and lack of 242 amino acids of the C- terminus. In each of these 4 families the mutations were shown to co-segregate with the disease phenotype, both by sequencing and by either size change, or alteration of a restriction endonuclease site. None of the mutations were found in Japanese or Caucasian control subjects. No other coding variants were found on sequencing the entire gene in each proband.

In the Australian family WS-5, a 1681C to T transversion (P504L) was observed. This mutation destroys a BsmFl restriction site. In Fig. 6C, the father (I-3) is shown to be heterozygous for 1681C to T, and the mother (I-4) is homozygous 1681C. Yet surprisingly all affected offspring (II-1, -2, and -4) appeared to be homozygous 1681T. The unaffected child (II-3) appeared to be homozygous for 1681C. The most likely explanation for these findings is that the mother's chromosome, inherited by each child (see haplotypes in Fig. 1E), harbored a microscopic deletion for the *WFS1* gene, and that the affected offspring were hemizygous for the P504L mutation.

A sixth family (WS-6) with one 10 year old affected child and two apparently unaffected younger sisters became available for analysis. The parents were Saudi Arabian first cousins. The affected child was homozygous for a 7bp repeat insertion at 1610 (CTGAAGG), resulting in a predicted frame shift and premature termination of the protein at codon 544. The parents were heterozygous, while the unaffected sisters were heterozygous and homozygous normal respectively. Sequence analysis also revealed a number of silent and intronic variants (polymorphisms) in various families (see Table 1).

Table 1. Mutations and polymorphisms in *WFS1*

Mutation	Amino acid	Exon/intron	Family	Control chromosomes
2812del(TC)	del882fs/ter937	Exon 8	WS-1	160[a]
1685del(CCTGCT CTATGTCTA)	del508YVYLL	Exon 8	WS-2	160 [a]
2341C to T	P724L	Exon 8	WS-3	160 [a]
2254G to T	G695V	Exon 8	WS-4	160[b]
2114G to A	W648X	Exon 8	WS-4	160[b]
1681 C to T	P504L	Exon 8	WS-5	160[b]
del*WFS1*[c]	---	---	WS-5	ND
1610insCTGAAGG	ins483fs/ter544	Exon 8	WS-6	160[d]
Changes of uncertain effect				
1167G to A	I333V	Exon 8	WS-4 (same chr. as W648X)	ND
Polymorphisms				
854G to C	D268D	Exon 6	WS-4	ND
1355T to C	V395V	Exon 8	WS-4	ND
1457C to T	C429C	Exon 8	WS-3	ND
1537A to G	R456H	Exon 8	WS-1,-2,-3,-4, normals	ND
1545C to T	L459L	Exon 8	WS-3	ND
1570T to C	L507L	Exon 8	WS-4	ND
1815C to T	L549L	Exon 8	WS-4	ND
1925C to T	F585F	Exon 8	WS-4	ND
2002G to A	H611R	Exon 8	WS-1,-2,-3, normals	ND
2603A to G	K811K	Exon 8	WS-4	ND
2735G to A	S855S	Exon 8	WS-4	ND
1032-5C to G		Intron 7	WS-1,-2,-3,-4	ND

a-Japanese
b-Caucasian
c-Not confirmed
d-Palestinian Arabs

Table 2. Primers for amplification, sequencing and mutation detection (SEQ ID NOS: 6-41, consecutively).

DNA Fragment	Primers	Product Size
Exon 1*	5'TGTAAAACGACGGCCAGTCTCGTGCAGAAGGCCGCGCT3' 5'CAGGAAACAGCTATGACCGCCCACAGCCACCGCGCCAC3'	247 bp
Exon 2	5'TGTAAAACGACGGCCAGTCTGTCTCCAGCAGACACTAA3' 5'CAGGAAACAGCTATGACCCACAATGCTGAACTGCAGAG3'	276 bp
Exon 3	5'TGTAAAACGACGGCCAGTCTGAAGACCCTCATGCCTTG3' 5'CAGGAAACAGCTATGACCACACTTCTCTGTGGGCTGTG3'	276 bp
Exon 4	5'TGTAAAACGACGGCCAGTTCGGAGAATCTGGAGGCTGA3' 5'CAGGAAACAGCTATGACCCATTACAAGCTGCTCAACCC3'	253 bp
Exon 5	5'TGTAAAACGACGGCCAGTCGAAAGCCTTCCAGGCAGAG3' 5'CAGGAAACAGCTATGACCCTATGGGAAGGTCCTGGCTC3'	353 bp
Exon 6	5'TGTAAAACGACGGCCAGTCTAGGAACAGTGCGCCAGTT3' 5'CAGGAAACAGCTATGACCATGGAGTCGCACAGGAAGGA3'	268 bp
Exon 7	5'TGTAAAACGACGGCCAGTGCCCATGCTGTTTTCTCTCA3' 5'CAGGAAACAGCTATGACCCCGAGGACACATCCTTATGA3'	371 bp
Exon 8a	5'TGTAAAACGACGGCCAGTCCTCGTTCCCACGTACCATC3' 5'CAGGAAACAGCTATGACCGTAGCAGTAGGTGCCCTTGA3'	766 bp
Exon 8b	5'TGTAAAACGACGGCCAGTCCTGGTCGTCCTCAATGTCA3' 5'CAGGAAACAGCTATGACCCATAGAACCAGCAGAACAGC3'	503 bp
Exon 8c	5'TGTAAAACGACGGCCAGTTGGTTCACGTCTCTGGAGCT3' 5'CAGGAAACAGCTATGACCGAGTTGTAGACCTTCATGCC3'	240 bp
Exon 8d*	5'TGTAAAACGACGGCCAGTGGGCATGAAGGTCTACAACT3' 5'CAGGAAACAGCTATGACCGAACTTCTTGATGTGGCAGG3'	362 bp
Exon 8e	5'TGTAAAACGACGGCCAGTCTGGATGCGCTGCCTCTACG3' 5'CAGGAAACAGCTATGACCTCAGGCCGCCGACAGGAATG3'	523 bp
Exon 8f	5'TGTAAAACGACGGCCAGTTCGCCTTCGACTTCTTTTTC3' 5'CAGGAAACAGCTATGACCCCAAACAAATAAGAAATGCT3'	499 bp
2812del(TC)	5' GCC CAG CTC TCG CCC ACC AG 3' 5' TCA GGC CGC CGA CAG GAA TG 3'	120 bp
1685del(N)$_{15}$	5' CCT GGT CGT CCT CAA TGT CA 3' 5' GGT AGG GCA CAA GGT AGC AG 3'	119 bp
2341C to T	EcoNI-F;5' GGGCATGAAGGTCTACAACTCCA 3' EcoNI-R;5' CCGTAGAGGCAGCGCATCCAGTCGCCGAcctAGAAC3'**	244 bp
2254G to T	5'-GAGGGCATGAAGGTCTACAA-3' 5'- CCCACGGTAATCTCAAACTT-3'	377 bp
2114G to A	5'-TAGTGTGCCCCTGCTGTTGC-3' 5'-CCCACGGTAATCTCAAACTT-3'	528 bp

* Due to the inability to directly sequence these PCR products, the fragments were subcloned into pGEM-T Easy Vector (Promega) as described by the manufacturer and several colonies sequenced for each individual.

** Because originally there was no appropriate restriction enzyme site to distinguish mutated alleles, the EcoNI-R primer was modified (3-base change, tga to cct, underlined in primer sequence) and a new EcoNI site was introduced to the mutated allele (CC2341(C/T)NNNNNAGG).

DISCUSSION

Consanguineous families from isolated regions of Japan provided the genetic material that led to the discovery of mutations in *WFS1* in WFS patients of diverse genetic backgrounds. We believe that mutant alleles at *WFS1* are responsible for the disease for several reasons, beyond the fact that the gene maps to the critical region. In each of the six pedigrees, mutant alleles of *WFS1* co-segregated with the disease phenotype. *WFS1* was shown to be expressed in brain, pancreatic islets, and in a β-cell insulinoma cell line, consistent with the disease phenotype. Seven different mutations were found, as well as a presumed microscopic deletion. The three missense mutations were evolutionarily conserved between the mouse and human (Figure 5), further suggesting their biological significance. None of the mutations were observed in normal chromosomes.

The Australian Caucasian family WS-5 was particularly interesting, as each affected child appeared to be homozygous for a P504L mutant allele inherited from the heterozygous father (Fig. 6C). Repeat sampling and analysis confirmed these results. Analysis of the mother's DNA with new markers between *D4S500* and *D4S431* suggested that the deletion was confined to a region of <170 kb. Recently a patient with another autosomal recessive disorder was observed to be heterozygous for a missense mutation in combination with a partial deletion of a gene (Ries et al., Human Mutation 12: 44-51, 1998).

The expression pattern of *WFS1* appeared ubiquitous by Northern analysis of polyA+ RNA (Fig. 3A). Yet interestingly, the most prominent mRNA observed in total RNA was that in pancreatic islets (Fig. 3C). This high level of expression of *WFS1* in islets might explain why the earliest manifestation of WFS is insulin-deficient diabetes mellitus (Barrett and Bundey, J Med Genet 34: 838-841, 1997). Further analysis of the cell biology of *WFS1* will be accomplished through generation

of specific antibodies, monitoring expression in cultured cells, and gene targeting to define genotype/phenotype relationships.

Swift et al. hypothesized that heterozygous carriers of the gene for WFS were 26-fold more likely to require psychiatric hospitalization than non-carriers (Swift et al., Molecular Psychiatry 3: 86-91, 1998). Blackwood et al (Blackwood et al., Nature Genetics 12:427-430, 1996) reported highest lod scores with markers mapping to the region *D4S431-D4S403* in a genome scan of a large family with bipolar affective disorder. These findings suggest that mutations in *WFS1* might be implicated in patients with psychiatric diseases.

Mutations in *WFS1* appear to result in premature death of pancreatic islet β-cells leading to juvenile onset insulin-requiring diabetes mellitus (Karasik et al., Diabetes Care 12: 135-138, 1989). The β-cell damage in autoimmune Type I diabetes likely results from the interaction of the HLA locus as a major susceptibility gene, along with multiple minor gene defects. In contrast, islet β-cell loss in WFS is monogenic in origin. Importantly, the *WFS1* gene appears to play a major role in maintaining normal islet β-cell function, as mutations in this gene alone can result in loss of islet β-cells. Genome scans for both Type I and Type II diabetes mellitus have not implicated major genes in the 4p region. Yet since these are complex diseases, mutations in *WFS1* might play a minor role in these more common forms of diabetes. In addition, *WFS1* may represent a new therapeutic target for treatment and prevention of diabetes mellitus and for neurodegenerative disorders.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

Claims

What is claimed:

1. A recombinant DNA molecule comprising a vector into which is inserted a heterologous DNA segment from human chromosome 4p, the segment being located between markers *D4S500* and *D4S431*, the segment comprising a gene, mutations of which are associated with Wolfram Syndrome.

2. The recombinant DNA molecule of claim 1, wherein the gene is composed of exons that form an open reading frame having a sequence that encodes a polypeptide about 880 to 900 amino acids in length.

3. The recombinant DNA molecule of claim 2, wherein the open reading frame encodes an amino acid having greater than 60% identity with SEQ ID NO: 3 or SEQ ID NO:5.

4. The recombinant DNA molecule of claim 4, wherein said open reading frame comprises a sequence having greater than 60% homology with SEQ ID NO:2 or SEQ ID NO: 4.

5. The recombinant DNA molecule of claim 1, wherein the gene is composed of exons having sequences greater than 60% homologous with the sequences of the corresponding exons in SEQ ID NO:1.

6. An oligonucleotide between about 10 and 100 nucleotides in length, which specifically hybridizes with a portion of the recombinant DNA molecule of claim 1.

7. An isolated nucleic acid molecule having a sequence that is part or all of a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) a variant of SEQ ID NO:1 that is substantially the same as SEQ ID NO:1 within the exons of SEQ ID NO:1;

c) a sequence having at least homology 60% to SEQ ID NO:1 within the exons of SEQ ID NO:1;

d) SEQ ID NO:2;

e) a variant of SEQ ID NO:2 that is substantially the same as SEQ ID NO:2;

f) a sequence having at least homology 60% to SEQ ID NO:2;

g) a sequence encoding a polypeptide substantially the same as SEQ ID NO:3;

h) a sequence encoding a polypeptide at least 60% homologous to SEQ ID NO:3;

i) a sequence encoding a polypeptide substantially the same as SEQ ID NO:3, that additionally comprises one or more of the sequence variants set forth in Table 1.

j) SEQ ID NO:4;

k) a variant of SEQ ID NO:4 that is substantially the same as SEQ ID NO:4;

l) a sequence having at least homology 60% to SEQ ID NO:4;

m) a sequence encoding a polypeptide substantially the same as SEQ ID NO:5; and

n) a sequence encoding a polypeptide at least 60% homologous to SEQ ID NO:5.

8. An oligonucleotide between 10 and 100 bases in length, that specifically hybridizes with a portion of the nucleic acid molecule of claim 7.

9. A polypeptide, which is produced by the expression of the nucleic acid molecule of claim 7.

10. Antibodies immunologically specific for the polypeptide of claim 9.

11. A polypeptide produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on human chromosome 4p between markers *D4S500* and *D4S431*, mutations of which are associated with Wolfram Syndrome.

12. The polypeptide of claim 11, which comprises a hydrophilic N-terminal region of about 300 amino acid residues, a hydrophilic C-terminal region of about 240 residues, and a central hydrophobic core of about 350 residues.

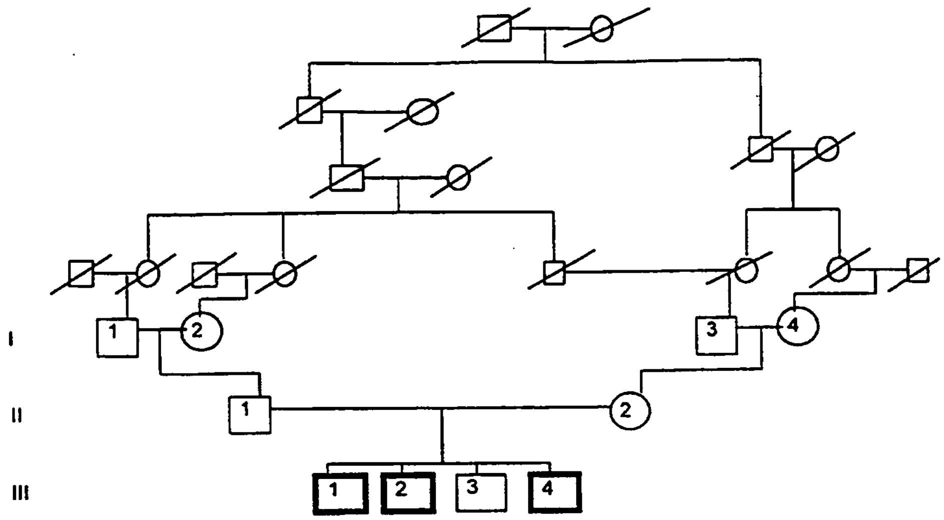
13. The polypeptide of claim 12, having an amino acid sequence substantially the same as part or all of SEQ ID NO: 3 or SEQ ID NO:5.

14. Antibodies immunologically specific for part or all of the polypeptide of claim 11.

15. A method for determining the predisposition of an individual to develop Wolfram Syndrome, which comprises examining a *WFS1* gene sequence of the individual for mutations resulting in expression of no gene product or a non-functional gene product, the mutations being indicative of the predisposition of the individual to develop Wolfram syndrome.

16. The method of claim 15, wherein the mutations comprises sequences selected from the group consisting of 2812del(TC), 1685del(CCTGCTCTATGTCTA), 2341C to T, 2254 G to T, 2114 G to A, 1681 C to T, and 1610ins(CTGAAGG).

17. The method of claim 16, wherein the mutations are detected by PCR amplification using primers selected from the group consisting of SEQ ID NOS: 32, 33, 34, 35, 36, 37, 38, 39, 40 and 41.



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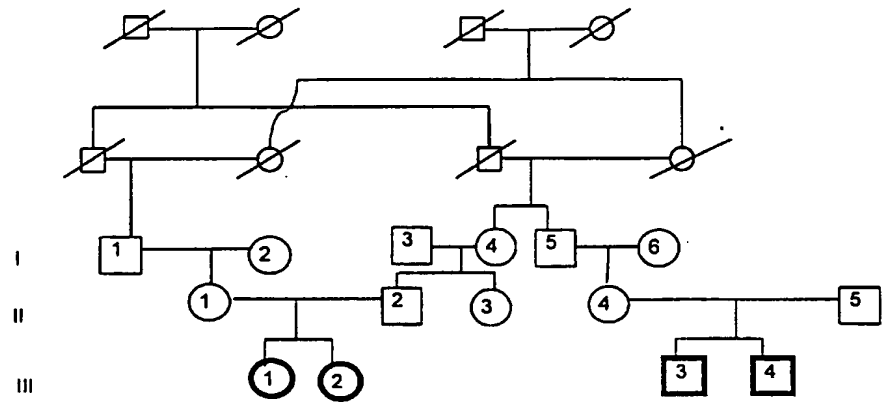
D4S412	4 3	1 3	4 3	4 4	4 1	3 1	4 4	4 1	4 1	2 1
D4S3023	9 5	3 4	9 4	9 9	9 7	4 9	9 9	9 7	9 7	7 7
D4S2925	2 2	1 1	2 1	2 2	2 3	1 2	2 2	2 3	2 3	2 3
Hox7	3 3	3 1	3 1	3 3	3 1	1 3	3 3	3 1	3 1	3 1
D4S2957	1 1	2 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1
D4S2375	1 2	2 4	1 4	1 1	1 3	4 1	1 1	1 3	1 3	2 3
A348XA5	6 2	6 1	6 1	6 6	6 6	1 6	6 6	6 6	6 6	3 6
D4S827	6 2	1 3	6 3	6 6	6 3	3 6	6 6	6 3	6 3	1 3
D4S500	1 5	2 6	1 6	1 1	1 1	6 1	1 1	1 1	1 1	1 1
D4S431	3 6	4 6	3 6	3 3	3 3	6 0	3 3	3 6	3 6	4 6
D4S2360	2 3	2 5	2 5	2 2	2 2	5 2	2 2	2 3	2 3	5 3
D4S2935	4 2	4 4	4 4	4 4	4 4	4 4	4 4	4 3	4 3	4 3
D4S3007	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 3	1 3	1 3
D4S394	3 1	1 1	3 1	3 3	3 3	1 3	3 3	3 3	3 3	1 3
D4S2983	1 3	2 1	1 1	1 3	1 3	1 3	1 3	3 5	3 5	1 5
D4S2923	1 3	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1

Figure 1a



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D4S3023	3	8	3	9	3	3	3	3	3	7	3	7	9	3	9	3	3	7	3	7	9	7
D4S2925	1	2	1	3	1	1	1	1	1	1	1	1	2	1	1	1	1	3	1	3	1	3
hox7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	3	1	3	3	1
D4S2957	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D4S2375	4	2	4	5	4	4	4	4	4	4	4	2	1	4	4	4	4	4	4	4	1	4
A348XA5	2	2	2	2	2	2	2	2	2	2	2	2	6	2	6	2	2	3	2	3	2	3
D4S827	4	4	4	4	4	4	4	4	4	6	4	5	6	4	6	4	4	6	4	6	4	6
D4S500	2	9	2	2	2	2	2	2	2	4	2	3	1	2	2	2	2	11	2	11	A	11
D4S431	6	4	6	6	6	6	6	6	6	6	6	6	3	6	4	6	6	6	6	6	4	6
D4S2360	4	2	4	5	4	4	4	4	4	5	4	5	2	4	5	4	4	4	4	4	1	4
D4S2935	2	4	2	4	2	2	2	2	2	3	2	3	4	2	4	2	2	2	2	2	2	2
D4S3007	3	1	3	3	3	3	3	3	3	3	3	1	1	3	1	3	3	3	3	3	1	3
D4S394	1	3	1	5	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1
D4S2983	1	1	1	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1
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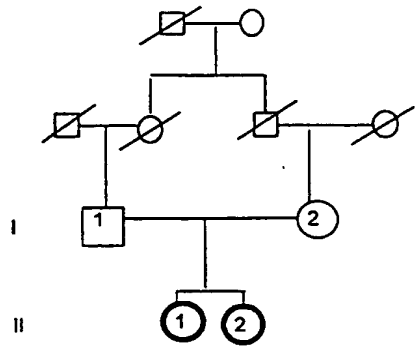
Figure 1b



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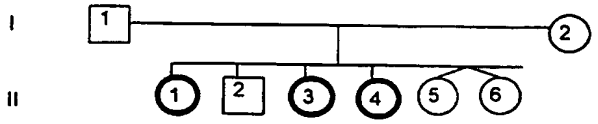
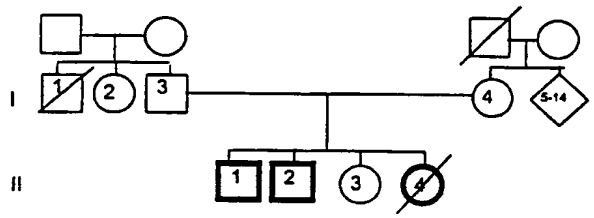
D4S412	2 2	2 2	2 2	4 2
D4S3023	5 6	6 6	6 6	9 6
D4S2925	3 1	1 1	1 1	2 1
Hox7	3 3	3 3	3 3	3 3
D4S2957	1 1	1 1	1 1	1 1
D4S2375	2 4	4 4	4 4	1 4
A348XA5	4 2	2 2	2 2	6 2
D4S827	4 6	6 6	6 6	6 6
D4S500	6 6	6 6	6 6	1 6
D4S431	1 4	4 4	4 4	3 4
D4S2360	5 3	3 3	3 3	2 3
D4S2935	4 4	4 4	4 4	4 4
D4S3007	3 3	3 3	3 3	1 3
D4S394	4 1	1 1	1 1	3 1
D4S2983	1 5	5 5	5 1	1 5
D4S2923	1 1	1 1	1 1	1 1

Figure 1c



D4S127	2 4	2 2	4 4	2 2	2 2	4 4	4 2	2 4
D4S412	3 4	3 3	4 1	3 3	3 3	4 1	4 3	3 1
D4S3023	2 1	2 3	2 7	2 3	2 3	1 7	1 3	3 7
D4S2925	3 3	3 3	3 1	3 3	3 3	3 1	3 3	3 1
hox7	1 1	1 2	1 3	1 2	1 2	1 3	1 2	2 3
D4S2957	3 1	3 3	3 1	3 3	3 3	1 1	1 3	3 1
D4S2375	4 6	4 2	4 2	4 2	4 2	6 2	6 2	2 2
A348XA5	1 3	1 5	1 1	1 5	1 5	3 1	3 5	5 1
D4S827	7 6	7 7	7 6	7 7	7 7	6 6	6 7	7 6
D4S500	7 1	7 2	7 7	7 2	7 2	1 7	1 2	2 7
D4S431	5 4	5 4	5 5	5 4	5 4	4 5	4 4	4 5
D4S2360	1 1	1 5	1 2	1 5	1 5	1 2	1 5	5 2
D4S2935	2 4	2 4	2 2	2 4	2 4	4 2	4 4	4 2
D4S3007	3 3	3 4	3 3	3 4	3 4	3 3	3 4	4 3
D4S394	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1
D4S2983	4 3	4 5	4 1	4 5	4 5	3 1	3 5	5 1
D4S2923	1 1	1 3	1 1	1 3	1 3	1 1	1 3	3 1

Figure 1d



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D4S1054	1 2	1 1	1 1	2 1	1 1	1 1
JW3	1 4	1 2	1 2	4 2	1 2	3 2
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Figure 1e

Figure 2a

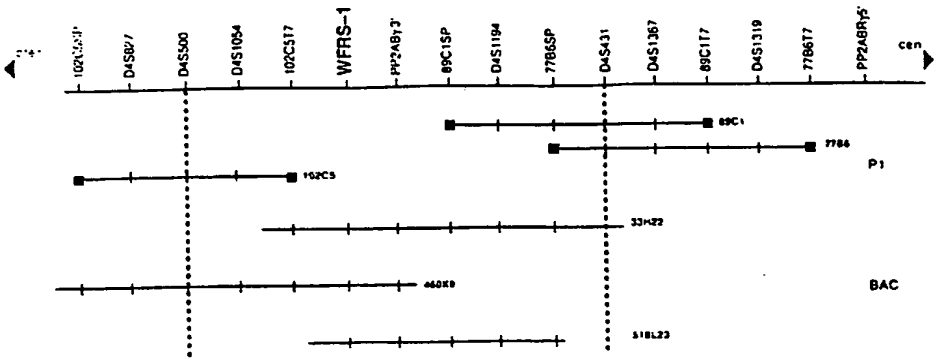


Figure 2b

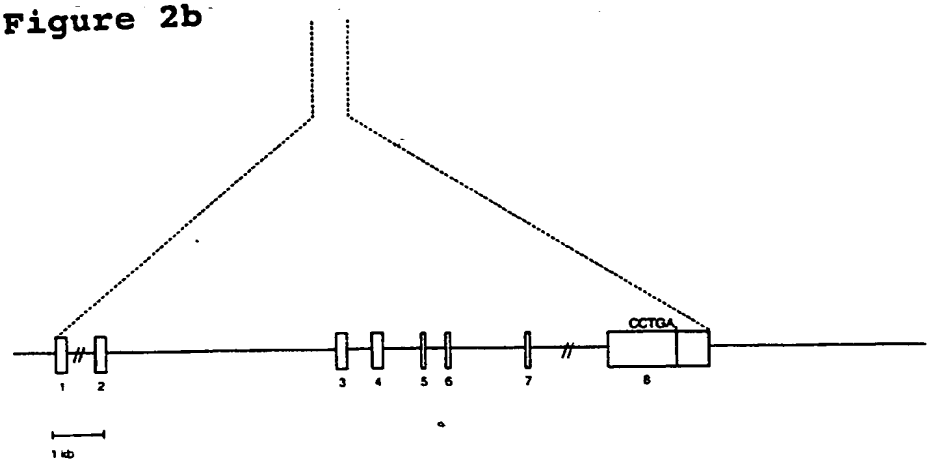


Figure 3a

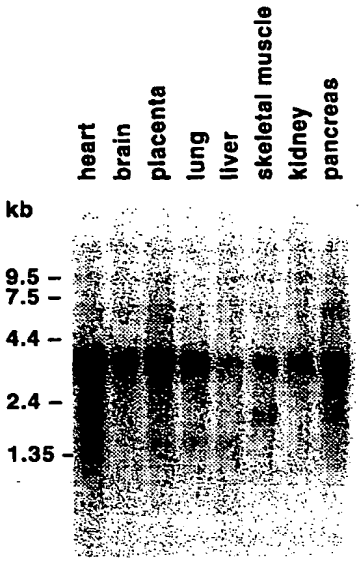


Figure 3b

Figure 3c

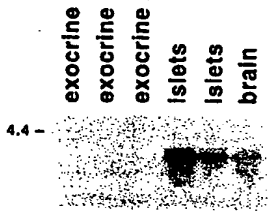


Figure 3d

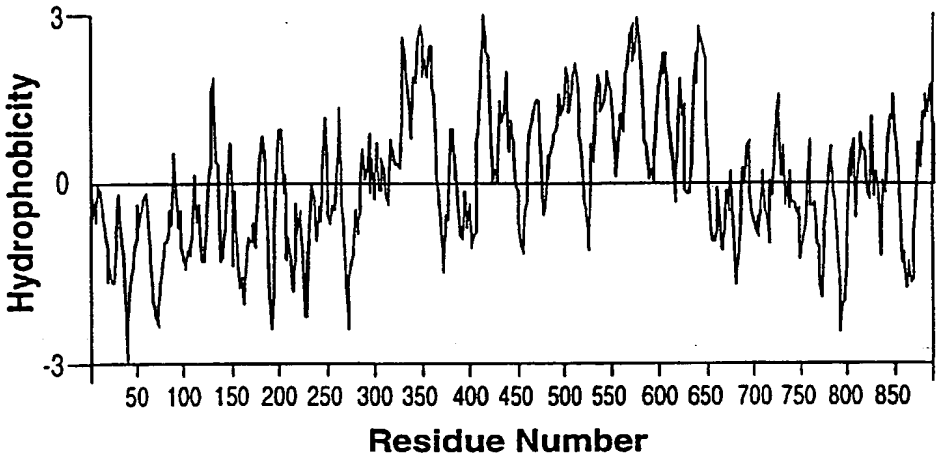
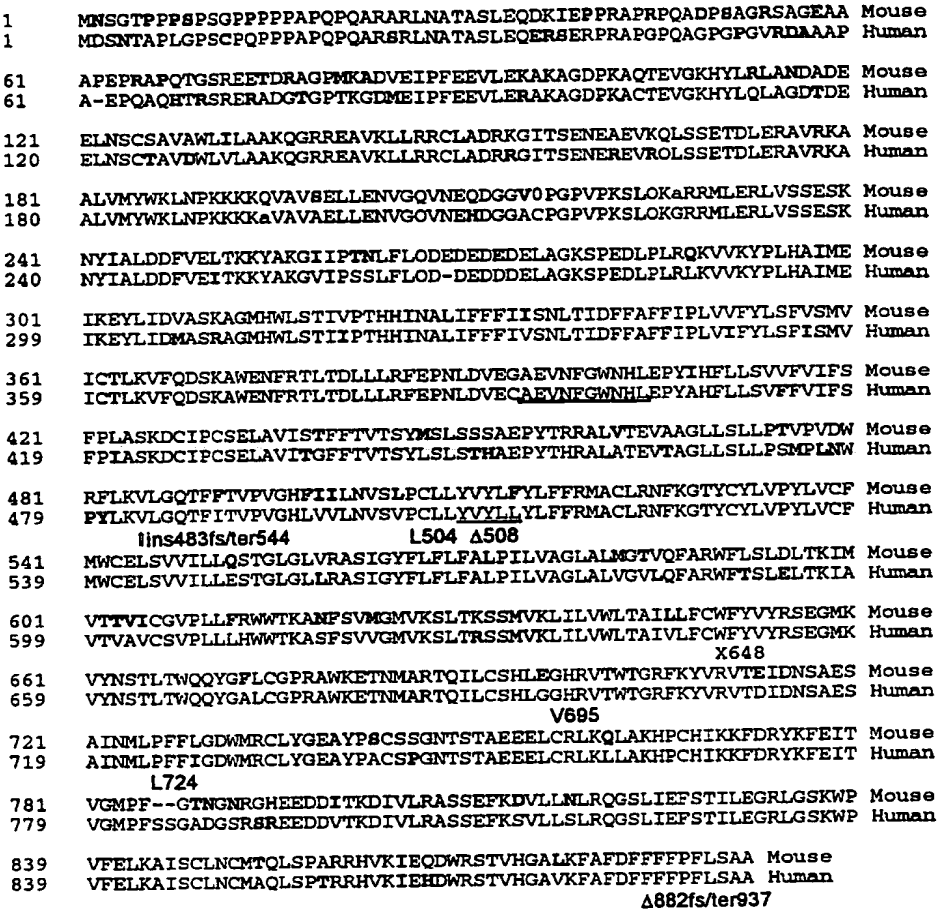

3
0
-3
Hydrophobicity
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850
Residue Number

Figure 4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601 VTTVICGVPLLFRWWTKANFSVMGMVKSLTKSSMVKLILVWLTAILLFCWFYVYRSEGMK Mouse
599 VTVAVCSVPLLLHWWTKASFSVVGMVKSLTRSSMVKLILVWLTAIVLFCWFYVYRSEGMK Human
X648
661 VYNSTLTWQQYGFLCGPRAWKETNMARTQILCSHLEGHRVTWTGRFKYVRVTEIDNSAES Mouse
659 VYNSTLTWQQYGALCGPRAWKETNMARTQILCSHLGGHRVTWTGRFKYVRVTDIDNSAES Human
V695
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779 VGMPFSSGADGSRSREEDDVTKDIVLRASSEFKSVLLSLRQGSLIEFSTILEGRLGSKWP Human
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Figure 5

Figure 6a

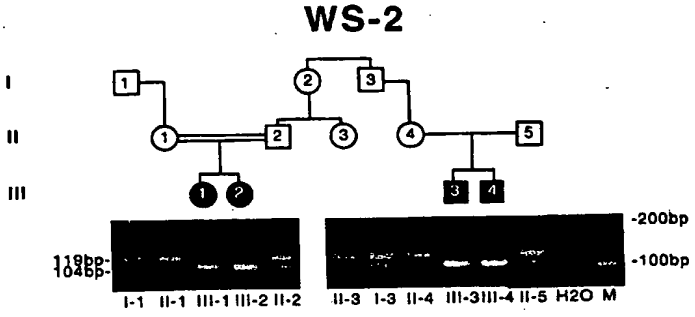


Figure 6b

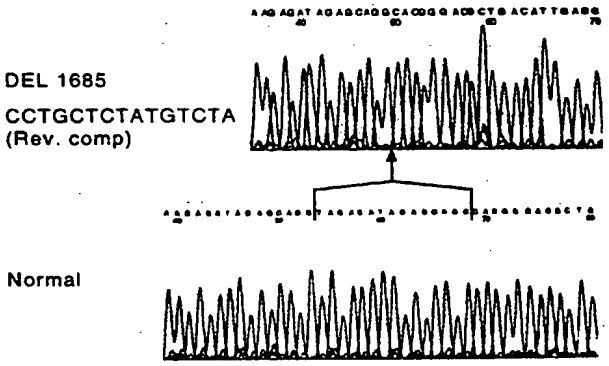
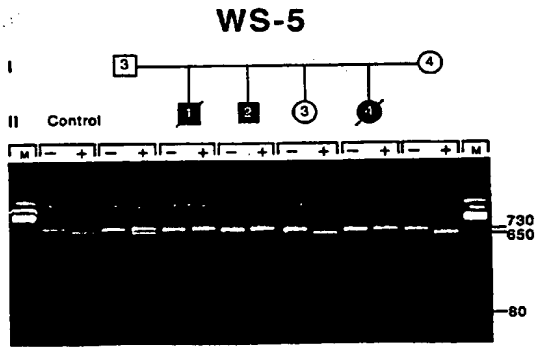


Figure 6c



SEQUENCE LISTING

<110> Permutt, M. Alan
Inoue, Hiroshi
Muekler, Mike

<120> Gene Mutated in Wolfram Syndrome

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<212> PRT
<213> Homo sapiens

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Leu Glu Gln Glu Arg Ser Glu Arg Pro Arg Ala Pro Gly Pro Gln Ala
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Gly Pro Gly Pro Gly Val Arg Asp Ala Ala Ala Pro Ala Glu Pro Gln
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Lys Gly Asp Met Glu Ile Pro Phe Glu Glu Val Leu Glu Arg Ala Lys
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Ala Gly Asp Pro Lys Ala Gln Thr Glu Val Gly Lys His Tyr Leu Gln
            100                 105                 110
Leu Ala Gly Asp Thr Asp Glu Glu Leu Asn Ser Cys Thr Ala Val Asp
        115                 120                 125
Trp Leu Val Leu Ala Ala Lys Gln Gly Arg Arg Glu Ala Val Lys Leu
    130                 135                 140
Leu Arg Arg Cys Leu Ala Asp Arg Arg Gly Ile Thr Ser Glu Asn Glu
145                 150                 155                 160
Arg Glu Val Arg Gln Leu Ser Ser Glu Thr Asp Leu Glu Arg Ala Val
                165                 170                 175
Arg Lys Ala Ala Leu Val Met Tyr Trp Lys Leu Asn Pro Lys Lys Lys
            180                 185                 190
Lys Gln Val Ala Val Ala Glu Leu Leu Glu Asn Val Gly Gln Val Asn
        195                 200                 205
Glu His Asp Gly Gly Ala Gln Pro Gly Pro Val Pro Lys Ser Leu Gln
    210                 215                 220
Lys Gln Arg Arg Met Leu Glu Arg Leu Val Ser Ser Glu Ser Lys Asn
225                 230                 235                 240
Tyr Ile Ala Leu Asp Asp Phe Val Glu Ile Thr Lys Lys Tyr Ala Lys
                245                 250                 255
Gly Val Ile Pro Ser Ser Leu Phe Leu Gln Asp Asp Glu Asp Asp Asp
            260                 265                 270
Glu Leu Ala Gly Lys Ser Pro Glu Asp Leu Pro Leu Arg Leu Lys Val
        275                 280                 285
Val Lys Tyr Pro Leu His Ala Ile Met Glu Ile Lys Glu Tyr Leu Ile
    290                 295                 300
Asp Met Ala Ser Arg Ala Gly Met His Trp Leu Ser Thr Ile Ile Pro
305                 310                 315                 320
Thr His His Ile Asn Ala Leu Ile Phe Phe Phe Ile Ile Ser Asn Leu
                325                 330                 335
Thr Ile Asp Phe Phe Ala Phe Phe Ile Pro Leu Val Ile Phe Tyr Leu
            340                 345                 350
Ser Phe Ile Ser Met Val Ile Cys Thr Leu Lys Val Phe Gln Asp Ser
        355                 360                 365
Lys Ala Trp Glu Asn Phe Arg Thr Leu Thr Asp Leu Leu Leu Arg Phe
    370                 375                 380
Glu Pro Asn Leu Asp Val Glu Gln Ala Glu Val Asn Phe Gly Trp Asn
385                 390                 395                 400
His Leu Glu Pro Tyr Ala His Phe Leu Leu Ser Val Phe Phe Val Ile
                405                 410                 415
Phe Ser Phe Pro Ile Ala Ser Lys Asp Cys Ile Pro Cys Ser Glu Leu
            420                 425                 430
Ala Val Ile Thr Gly Phe Phe Thr Val Thr Ser Tyr Leu Ser Leu Ser
        435                 440                 445
Thr His Ala Glu Pro Tyr Thr Arg Arg Ala Leu Ala Thr Glu Val Thr
    450                 455                 460
Ala Gly Leu Leu Ser Leu Leu Pro Ser Met Pro Leu Asn Trp Pro Tyr
465                 470                 475                 480
Leu Lys Val Leu Gly Gln Thr Phe Ile Thr Val Pro Val Gly His Leu
                485                 490                 495
Val Val Leu Asn Val Ser Val Pro Cys Leu Leu Tyr Val Tyr Leu Leu
            500                 505                 510
Tyr Leu Phe Phe Arg Met Ala Gln Leu Arg Asn Phe Lys Gly Thr Tyr
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Cys Tyr Leu Val Pro Tyr Leu Val Cys Phe Met Trp Cys Glu Leu Ser
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Val Val Ile Leu Leu Glu Ser Thr Gly Leu Gly Leu Leu Arg Ala Ser
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Ile Gly Tyr Phe Leu Phe Leu Phe Ala Leu Pro Ile Leu Val Ala Gly
                565                 570                 575
Leu Ala Leu Val Gly Val Leu Gln Phe Ala Arg Trp Phe Thr Ser Leu
            580                 585                 590
Glu Leu Thr Lys Ile Ala Val Thr Val Ala Val Cys Ser Val Pro Leu
        595                 600                 605
Leu Leu His Trp Trp Thr Lys Ala Ser Phe Ser Val Val Gly Met Val
    610                 615                 620
Lys Ser Leu Thr Arg Ser Ser Met Val Lys Leu Ile Leu Val Trp Leu
625                 630                 635                 640
Thr Ala Ile Val Leu Phe Cys Trp Phe Tyr Val Tyr Arg Ser Glu Gly
                645                 650                 655
Met Lys Val Tyr Asn Ser Thr Leu Thr Trp Gln Gln Tyr Gly Ala Leu
            660                 665                 670
Cys Gly Pro Arg Ala Trp Lys Glu Thr Asn Met Ala Arg Thr Gln Ile
        675                 680                 685
Leu Cys Ser His Leu Glu Gly His Arg Val Thr Trp Thr Gly Arg Phe
    690                 695                 700
Lys Tyr Val Arg Val Thr Asp Ile Asp Asn Ser Ala Glu Ser Ala Ile
705                 710                 715                 720
Asn Met Leu Pro Phe Phe Ile Gly Asp Trp Met Arg Cys Leu Tyr Gly
                725                 730                 735
Glu Ala Tyr Pro Ala Cys Ser Pro Gly Asn Thr Ser Thr Ala Glu Glu
            740                 745                 750
Glu Leu Cys Arg Leu Lys Leu Leu Ala Lys His Pro Cys His Ile Lys
        755                 760                 765
Lys Phe Asp Arg Tyr Lys Phe Glu Ile Thr Val Gly Met Pro Phe Ser
    770                 775                 780
Ser Gly Ala Asp Gly Ser Arg Ser Arg Glu Glu Asp Asp Val Thr Lys
785                 790                 795                 800
Asp Ile Val Leu Arg Ala Ser Ser Glu Phe Lys Ser Val Leu Leu Ser
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Leu Arg Gln Gly Ser Leu Ile Glu Phe Ser Thr Ile Leu Glu Gly Arg
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Leu Gly Ser Lys Trp Pro Val Phe Glu Leu Lys Ala Ile Ser Cys Leu
        835                 840                 845
Asn Cys Met Ala Gln Leu Ser Pro Thr Arg Arg His Val Lys Ile Glu
    850                 855                 860
His Asp Trp Arg Ser Thr Val His Gly Ala Val Lys Phe Ala Phe Asp
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Phe Phe Phe Phe Pro Phe Leu Ser Ala Ala
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Leu Glu Gln Asp Lys Ile Glu Pro Pro Arg Ala Pro Arg Pro Gln Ala
        35                  40                  45
Asp Pro Ser Ala Gly Arg Ser Ala Gly Glu Ala Ala Ala Pro Glu Pro
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    50                  55                  60
Arg Ala Pro Gln Thr Gly Ser Arg Glu Glu Thr Asp Arg Ala Gly Pro
65                  70                  75                  80
Met Lys Ala Asp Val Glu Ile Pro Phe Glu Glu Val Leu Glu Lys Ala
                85                  90                  95
Lys Ala Gly Asp Pro Lys Ala Gln Thr Glu Val Gly Lys His Tyr Leu
            100                 105                 110
Arg Leu Ala Asn Asp Ala Asp Glu Glu Leu Asn Ser Cys Ser Ala Val
        115                 120                 125
Ala Trp Leu Ile Leu Ala Ala Lys Gln Gly Arg Arg Glu Ala Val Lys
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Leu Leu Arg Arg Cys Leu Ala Asp Arg Lys Gly Ile Thr Ser Glu Asn
145                 150                 155                 160
Glu Ala Glu Val Lys Gln Leu Ser Ser Glu Thr Asp Leu Glu Arg Ala
                165                 170                 175
Val Arg Lys Ala Ala Leu Val Met Tyr Trp Lys Leu Asn Pro Lys Lys
            180                 185                 190
Lys Lys Gln Val Ala Val Ser Glu Leu Leu Glu Asn Val Gly Gln Val
        195                 200                 205
Asn Glu Gln Asp Gly Gly Val Gln Pro Gly Pro Val Pro Lys Ser Leu
    210                 215                 220
Gln Lys Gln Arg Arg Met Leu Glu Arg Leu Val Ser Ser Glu Ser Lys
225                 230                 235                 240
Asn Tyr Ile Ala Leu Asp Asp Phe Val Glu Leu Thr Lys Lys Tyr Ala
                245                 250                 255
Lys Gly Ile Ile Pro Thr Asn Leu Phe Leu Gln Asp Glu Asp Glu Asp
            260                 265                 270
Glu Asp Glu Leu Ala Gly Lys Ser Pro Glu Asp Leu Pro Leu Arg Gln
        275                 280                 285
Lys Val Val Lys Tyr Pro Leu His Ala Ile Met Glu Ile Lys Glu Tyr
    290                 295                 300
Leu Ile Asp Val Ala Ser Lys Ala Gly Met His Trp Leu Ser Thr Ile
305                 310                 315                 320
Val Pro Thr His His Ile Asn Ala Leu Ile Phe Phe Phe Ile Ile Ser
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Tyr Leu Ser Phe Val Ser Met Val Ile Cys Thr Leu Lys Val Phe Gln
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Asp Ser Lys Ala Trp Glu Asn Phe Arg Thr Leu Thr Asp Leu Leu Leu
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Arg Phe Glu Pro Asn Leu Asp Val Glu Gln Ala Glu Val Asn Phe Gly
385                 390                 395                 400
Trp Asn His Leu Glu Pro Tyr Ile His Phe Leu Leu Ser Val Val Phe
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Val Ile Phe Ser Phe Pro Leu Ala Ser Lys Asp Cys Ile Pro Cys Ser
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Glu Leu Ala Val Ile Ser Thr Phe Phe Thr Val Thr Ser Tyr Met Ser
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Arg Phe Leu Lys Val Leu Gly Gln Thr Phe Phe Thr Val Pro Val Gly
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His Phe Ile Ile Leu Asn Val Ser Leu Pro Cys Leu Leu Tyr Val Tyr
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Leu Phe Tyr Leu Phe Phe Arg Met Ala Gln Leu Arg Asn Phe Lys Gly
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Ala Gly Leu Ala Leu Met Gly Thr Val Gln Phe Ala Arg Trp Phe Leu
            580                 585                 590
Ser Leu Asp Leu Thr Lys Ile Met Val Thr Thr Val Ile Cys Gly Val
        595                 600                 605
Pro Leu Leu Phe Arg Trp Trp Thr Lys Ala Asn Phe Ser Val Met Gly
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Met Val Lys Ser Leu Thr Lys Ser Ser Met Val Lys Leu Ile Leu Val
625                 630                 635                 640
Trp Leu Thr Ala Ile Leu Leu Phe Cys Trp Phe Tyr Val Tyr Arg Ser
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Glu Gly Met Lys Val Tyr Asn Ser Thr Leu Thr Trp Gln Gln Tyr Gly
            660                 665                 670
Phe Leu Cys Gly Pro Arg Ala Trp Lys Glu Thr Asn Met Ala Arg Thr
        675                 680                 685
Gln Ile Leu Cys Ser His Leu Glu Gly His Arg Val Thr Trp Thr Gly
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Arg Phe Lys Tyr Val Arg Val Thr Glu Ile Asp Asn Ser Ala Glu Ser
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Ala Ile Asn Met Leu Pro Phe Phe Leu Gly Asp Trp Met Arg Cys Leu
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Tyr Gly Glu Ala Tyr Pro Ser Cys Ser Ser Gly Asn Thr Ser Thr Ala
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Ile Lys Lys Phe Asp Arg Tyr Lys Phe Glu Ile Thr Val Gly Met Pro
    770                 775                 780
Phe Gly Thr Asn Gly Asn Arg Gly His Glu Glu Asp Asp Ile Thr Lys
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22429

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/04; C07K 14/46, 16/28; C12Q 1/68; C12P 19/34
US CL :536/23.5, 24.31; 530/350, 387.9; 435/6, 91.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5, 24.31; 530/350, 387.9; 435/6, 91.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE
search terms:wolfram syndrome

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T,E	HARDY et al. Clinical and molecular genetic analysis of 19 Wolfram syndrome kindreds demonstrating a wide spectrum of mutations in WFS1. American Journal of Human Genetics. November 1999, Vol. 65, No. 5, pages 1279-1290.	1-17
A	COLLIER et al. Linkage of Wolfram syndrome to chromosome 4p16.1 and evidence for heterogeneity. American Journal of Human Genetics. October 1996, Vol. 59, No. 4, pages 855-863.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 29 JANUARY 2000	Date of mailing of the international search report 11 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID S. ROMEO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/22429

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	STROM et al. Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin) coding for a predicted transmembrane protein. Human Molecular Genetics. December 1998, Vol. 7, No. 13, pages 2021-2028, see entire document.	1-17
P, X	INOUE et al. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). Nature Genetics, October 1998, Vol. 20, No. 2, pages 143-148, see entire document.	1-17
X	US 5,578,444 A (EDWARDS et al.) 26 November 1996, columns 115-118, SEQ ID NO:36.	6-8